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Biochimica et Biophysica Acta 1762 (2006) 1 – 16
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Lipopolysaccharides of *Vibrio cholerae*: III. Biological functions

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Received 15 April 2005; received in revised form 15 August 2005; accepted 15 August 2005
Available online 2 September 2005

Abstract

This review presents the salient features of the biological functions including the (i) endotoxic activities, (ii) antigenic properties, (iii) immunological responses to and (iv) phage receptor activities of the *Vibrio cholerae* lipopolysaccharides (LPS). The biological functions of the capsular polysaccharide (CPS) of *V. cholerae* have also been discussed briefly as a relevant topic. The roles of LPS and other extracellular polysaccharides in the (i) intestinal adherence and virulence of the vibrios and (ii) the biofilm formation by the organisms have been analysed on the basis of the available data. Every effort has been made to bring out, wherever applicable, the lacunae in our knowledge. The need for the continuous serogroup surveillance and monitoring of the environmental waters and the role of LPS in the designing of newer cholera vaccines has been discussed briefly in conclusion.

Keywords: *Vibrio cholerae*; Lipopolysaccharide (LPS); Capsular polysaccharide (CPS); Endotoxin; Antigen (Ag); Antibody (Ab); Immunoglobulin (Ig); Phage receptor; Biofilm; Vaccine

1. Introduction

The physical and chemical characterization of the *Vibrio cholerae* LPS followed by an in-depth study of the genetics of its biosynthesis has recently been reviewed [1,2]. The O-Ag polysaccharide (O-PS) has figured out as an important constituent of the *V. cholerae* LPS. These analyses have pointed out the significant role of *V. cholerae* LPS in the causation and spread of the disease, cholera. As a logical follow up of these studies, the biological functions of the *V. cholerae* LPS and of its different constituents, particularly in relation to the causation and spread of the cholera epidemics, have naturally drawn our attention. While lipid-A is the key constituent exhibiting endotoxic properties, the O-PS of *V. cholerae* LPS is mainly responsible for its immunogenicity and production of vibriocidal antibodies in the host, and has led to the designing of different types of conjugated or non-conjugated cholera vaccines. An account of these functional aspects of *V. cholerae* LPS has been presented in this review. This review has further taken the opportunity to discuss the role of *V. cholerae* LPS (i) in the formation of biofilm that enables the organism to survive in the hostile natural environments, (ii) in the intestinal adherence and colonization of the organisms as an important step in the causation of the disease in the infected host and (iii) as receptor of cholera phages in the control of bacterial population in the aquatic environments. The role of CPS associated with *V. cholerae* of several serogroups in the expression of virulence of the organisms has also been briefly outlined. The abbreviations and nomenclatures used in the earlier two reviews [1,2] will be used here as such and unless stated otherwise for any particular case.

2. Endotoxic activities

LPSs of many Gram-negative bacteria were known to exhibit a wide spectrum of endotoxic activities [3]. In conformity with this, the LPS of *V. cholerae* was shown to exhibit several endotoxic activities, e.g., pyrogenicity, lethality to mice, local Shwartzmann reaction and limulus lysate gelation [4,5]. *V. cholerae* LPS was also shown to exhibit mitogenic effects and possess adjuvant properties [5]. It induced in vitro proliferation of murine spleen lymphocytes

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doi:10.1016/j.bbadis.2005.08.005

and also murine intestinal lymphocytes as measured by the uptake of (^{3}H) thymidine [5].

2.1. Role of lipid-A

Studies on different Gram-negative bacteria have shown that among all the chemical constituents of LPS, lipid-A is mostly responsible for its endotoxic activities [6,7]. Chemical isolation of lipid-A confirmed that it was the active domain responsible for the induction of all known pathophysiological LPS effects [8,9]. Since lipid-A of *V. cholerae* LPS possesses general structural similarity with the lipid-A of many other Gram-negative bacteria [10], it is expected that the *V. cholerae* lipid-A would behave similarly. Lipid-A obtained from five different strains of *V. cholerae* and complexed with bovine serum albumin (BSA) did in fact exhibit the following endotoxic activities: (i) bone marrow reaction, (ii) limulus lysate gelation, (iii) pyrogenicity, (iv) mouse/chick embryo lethality, (v) tumor hemorrhage and (vi) complement inactivation [11]. Experiments conducted in parallel with LPS and lipid-A of these strains revealed that lipid-A was the major contributing factor of the endotoxic properties of LPS and that lipid-A–BSA complexes were comparatively slightly more active than the parental LPSs in many of the tests carried out [11].

2.2. Role of any constituent chemical group

The specific chemical modification of endotoxins had proved to be a very efficient tool in recognizing the participation of a particular group in their toxic activities [12,13]. In one set of experiments [14], LPS of *V. cholerae* was treated separately with succinic anhydride, phthalic anhydride (both estimated to produce significant decrease in fatty acids, particularly the ester-linked fatty acids) and dinitrophenyl ethylene diamine (producing increase in total fatty acid content) and the resulting changes, if any, of the endotoxic activities, e.g., lethal activities in chick embryos and mice and local Shwartzmann reaction in rabbits, were noted. These studies indicated that the ester-linked fatty acids and in particular 3-hydroxy lauric acid in LPS of *V. cholerae* played a crucial role in eliciting some of its toxic effects. This was in conformity with the finding that in Gram-negative bacteria the ester-linked fatty acids were found, in general, as the important factors determining the toxicity of LPS [3,7,15]. The succinyl and phthalyl derivatives of LPSs from *S. typhimurium* and *S. minnesota* were less toxic in mice than the original LPS [13]. In *E. coli*, succinylation and phthalylation of LPSs reduced the toxicity and pyrogenicity without any considerable loss in ester-linked fatty acids [12]. Rietschel et al. [16], however, showed that succinylation of glycolipids from *S. minnesota* R595 had no effect on pyrogenicity as well as lethality in animals. Alkali digested LPSs from *V. cholerae* O1 with a loss in ester-linked fatty acids were nontoxic to rabbits and mice [4]. In a different set of experiments, mutants of *V. cholerae* 569 B resistant to common antibiotics and neutral and anionic detergents were isolated [17]. The outer membranes of these strains showed a significant deficiency in the acylation of lipid-A. The contents of amide- and ester-linked fatty acids in the lipid-A of these strains were reduced to 50–56% and 29–37%, respectively. This defect was specific for lipid-A as there was no change in the acylation of phospholipids. The reduction in the fatty acid content of lipid-A was reflected in the endotoxic properties of LPSs of these mutant strains. LPS from both mutant strains exhibited markedly low endotoxicity in the localized Shwartzmann reaction, limulus gelation assay and in the complement fixation assay. All these studies indicated the role of fatty acids in the endotoxicity of LPS and ruled out the possibility of involvement of any particular chemical constituent of LPS in all its endotoxic activities.

2.3. Effect on cell morphology

LPSs from many different enteric bacteria were found to alter human endothelial cell morphology in vitro in a species-dependent and dose-dependent manner [18] and were therefore likely to contribute to the vascular pathology of Gram-negative infections. Seifert et al. [18] observed that LPSs derived from *V. cholerae* cells produced no alteration of human endothelial cell morphology. But Islam et al. [19] directly added endotoxins from different enteric pathogens, including *V. cholerae* Inaba 569 B, to the neutrophils in suspension and observed that each one of the endotoxins tested changed the shape of the neutrophils. The relevance of these findings to the human infection with *V. cholerae* is not immediately obvious.

2.4. Effect on neutrophil chemotaxis

While the endotoxins themselves were not found to be chemotactic [20], they were found to induce chemotactic activity in plasma by the activation of complement [21]. Bignold et al. [22] showed that endotoxins from various bacteria, including *V. cholerae*, inhibited chemotaxis of neutrophils to IL-8. No endotoxin affected chemotaxis to formyl peptide or was itself chemotactic for neutrophils. It was suggested that chemotaxis to IL-8 might be mediated by cellular mechanisms different from those involved in chemotaxis to formyl peptide. Islam et al. [19] directly added endotoxins from different enteric pathogens, including *V. cholerae Inaba 569 B*, to the neutrophils in suspension and found that each one of them stimulated the neutrophils to acquire locomotor morphology. Based on the earlier observations on the responses of neutrophils [23] and monocytes [24] to chemotactic factors, the authors concluded that the endotoxins acted as chemotactic factors.

2.5. Effect on haemagglutinating activity of bacterial cells

In cases of several human pathogens, the haemagglutinating ability of bacterial cells was closely correlated with the bacterial ability to adhere to the host intestine [25,26]. Alam et al. [27] found that haemagglutination was a common function of the polysaccharide moiety of LPSs from important human enteropathogenic bacteria including *V. cholerae* O139 Bengal. *V. cholerae* O139 LPS showed the highest haemag-

glutinating activity. The authors [27] argued that since cell-mediated haemagglutination was correlated with bacterial adherence, haemagglutination induced by the polysaccharide moiety of LPS indicated that LPS was a potential adhesin.

3. Antigenic properties

The LPS molecule contains three distinct regions: the lipid A region which is hydrophobic and forms part of the lipid bilayer of the outer membrane, the core oligosaccharide, and the O-Ag. The outermost region, the O-Ag, provides the major antigenic variability of the cell surface. There are several classification systems for the O-Ag of *V. cholerae* [28]. The typing scheme of Sakazaki and Shimada [29] is the most widely used system and uses sera against heat- killed organisms. Their scheme involved 138 different serogroups. The serogroup O139 and others beyond 139 were added to it subsequently.

V. cholerae O1 has been divided into two biotypes, Classical and El Tor, which are further subdivided into three serotypes: Inaba, Ogawa and Hikojima. The three serotypes have been distinguished on the basis of three antigenic determinants or epitopes, A, B and C, associated with the O-Ag of the LPS and defined using cross-absorbed antisera [30–32]. These epitopes are absent in rough mutants lacking the O-Ag [33–35]. All three serotypes share a common epitope, A. Inaba strains express A and C epitopes, whereas the Ogawa and Hikojima serotypes express A, B and a lesser amount of the C epitope [31,36]. The Hikojima subtype is rare and unstable, is not recognized by many investigators and rather appears to be a variant of Ogawa serotype [1,37,38]. Analysis of the chemical structure of the O-Ag has shown it to be composed of a homopolymer containing the amino sugar D-perosamine substituted with 3-deoxy-L-glycerotetronic acid, which may be the A epitope [28,37] present in both Inaba and Ogawa serotypes. Gustafsson and Holme [33] carried out some immunochemical studies and gel permeation chromatography of the polysaccharide fractions extracted from the Ogawa, Inaba and Hikojima serotypes of *V. cholerae* O1 strains. The authors showed that the A epitope was present as multiple determinants, supporting the view that the perosamine polymer was the structural basis for this epitope, and that the B and C epitopes were present as single determinants on each polysaccharide chain. Wang et al. [39] carried out binding studies of anti-Ogawa Abs IgG1 S-20-6 and IgG1 S-20-4 with synthetic methyl α-glycosides of fragments (up to the hexasaccharide) of the Ogawa O-PS, as well as with analogs of the terminal monosaccharide, and revealed that the terminal residue accounted for approximately 90% of the maximal binding energy. Their study showed that the terminal monosaccharide of the O-Ag of Ogawa LPS, bearing the 2-O-methyl group, was probably the serotype-specific determinant for the Ogawa strain, i.e., the B epitope. Further, binding studies with monoclonal Ab, that recognized an epitope common to both Ogawa and Inaba serotypes, suggested that it partially recognized the core and the O-PS of the LPS [39]. The epitope that is common to the Ogawa and Inaba serotypes was further characterized by purifying the core and the O-PS linked to the core of *V. cholerae* O1 LPS by preparative electrophoresis [40]. The O-PS was then subjected to periodate oxidation to destroy sugars. ELISA studies of the binding of these purified saccharide fragments to a monoclonal Ab, IgG3, that recognized both the Ogawa and Inaba serotypes, showed that both the core and the O-PS were involved in this common epitope, which was supposed to be the C-epitope. In order to understand the structural basis of carbohydrate recognition and the *V. cholerae* serotype specificity, Villeneuve et al. [41] undertook crystallographic studies of protective anti-cholera Abs in complex with synthetic analogs of the O-Ag. The crystal structure of the murine Fab S-20-4 from a protective Ab specific for the LPS Ag of the Ogawa serotype was determined in its unliganded form and in complex with synthetic fragments of the Ogawa O-PS. The authors presented a model of the Ab-O-PS complex based on the previously determined structure of the Fab–disaccharide complex. This model (Fig. 1) showed that the upstream terminal perosamine is bound inside the Ab-binding cavity, the 2-O-methyl group lying at the centre of the interface. The second perosamine residue is positioned at the exterior of the binding site and makes fewer contacts with the Ab residues, whereas subsequent sugar residues are not involved in the interaction. This study thus confirmed that the upstream terminal monosaccharide of the O-PS was the primary antigenic determinant or epitope and explained the serotype specificity of anti-Ogawa Abs by showing the pivotal contribution by so small a structural fragment in the antigenic determinant as a methyl group.

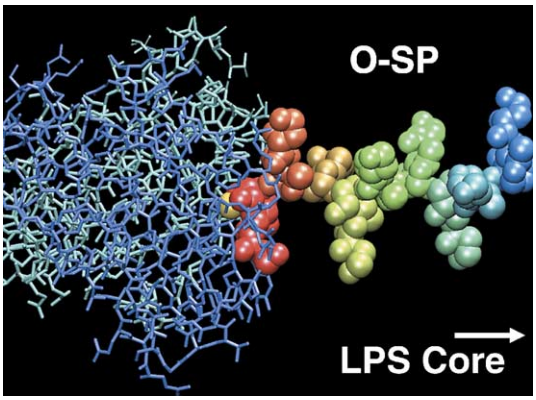

Fig. 1. Methylated-α-glycoside of the disaccharide of *V. cholerae* O1 O-PS, serotype Ogawa, was synthesized, complexed with the Fab fragment of monoclonal mouse Ab S-20-4 specific for the LPS Ag of the Ogawa serotype and crystallized. The figure above shows the model of the Ab-O-PS complex derived from X-ray crystallographic study of Fab–disaccharide complex. Individual perosamine residues are shown in different colors; the light and heavy chains of the Ab variable domains are shown in light and dark blue, respectively. The upstream terminal perosamine is bound inside the Ab-binding cavity (the 2-O-methyl group at the center of the interface is shown in yellow). Reproduced from the paper of Villeneuve et al. [41] with permission from PNAS, USA.

4. Immunological responses

4.1. Vibriocidal antibody level and immunity

LPSs of enteric pathogens are immunogenic and appear to be the primary protective Ags [42–45]. *V. cholerae* O1 LPS

was shown to induce protective immune responses in humans and animals [43,46–52] and thus its use as a protective immunogen for cholera vaccine development has been widely accepted [51,53,54]. The gene clusters that determined the biosynthesis of the LPS O-Ag of *V. cholerae* O1, of both the Inaba and Ogawa serotypes, were cloned and expressed in *E. coli* K-12 [44]. The O-Ags expressed by the modified *E.coli* K-12 had the specificity of *V. cholerae*. Abs raised against *E.coli* K-12 that harbored one of these clones, were as highly protective in the infant mouse model system as were Abs to *V. cholerae* itself. This study showed that the O-PS is the protective antigen in LPS of *V. cholerae*. Abs to these Ags are generally measured using a vibriocidal assay [55,56], which records killing of *V. cholerae* cells in presence of immune sera and complement. Mosley [53,57] provided evidence for the involvement of systemic Abs in protection against cholera and demonstrated a correlation between serum vibriocidal Ab level and protection against cholera. Elevated vibriocidal Ab levels were correlated with protection against both *V. cholerae* O1 colonization and disease [58]. The titer of vibriocidal Abs increased after natural infection with *V. cholerae* O1 or O139 [59,60] and after oral or parenteral vaccination [61,62]. The field trials of vaccines showed that the level of vibriocidal antibodies in serum was the best measure of induced immunity, since it correlated with the elicitation of a protective intestinal immune response against cholera [63,64]. The majority of vibriocidal antibodies were absorbed with *V. cholerae* LPS. It was, however, suggested by others that vibriocidal Abs were simply markers of protection and other Abs, probably of local origin, provided protection [65]. However, similar to protection against experimental cholera caused by *V. cholerae* O1 bacteria [54], a strong synergistic protective effect was achieved when anti-O139 bacterial Abs and anti-CT(cholera toxin)B (subunit B) Abs were combined even though the antitoxic immunity by itself was only marginally effective [47]. The mechanism of such synergistic action is, however, not known.

4.2. Monoclonal antibodies

MAbs directed against *V. cholerae* O1 were found to recognize the polysaccharide moiety of the corresponding LPS [66], to be vibriocidal and to agglutinate *V. cholerae* O1 strains. When subcutaneously injected into neonatal mice, these Abs protected mice against an oral challenge with *V. cholerae* O1 [66]. Protection was serotype dependent. LPS is a type 1 T cell-independent Ag. In cases of some other pathogenic organisms, it was shown that at high levels, purified LPS was mitogenic for mouse B cells, resulting in panIg production. At low levels, LPS could induce LPS-specific IgM and IgG Abs in humans and mice when the appropriate conditions were present [67,68]. In the case of *V. cholerae* cells, LPS could induce isotype switching to IgG and IgA Abs that were protective [49,51, 54,69]. The exact conditions needed for anti O-PS Ab responses to form in humans with cholera have not been defined. Anti-LPS IgM was thought to be operative in in vitro vibriocidal assays in which a positive response correlated with clinical protection in humans [48]. These Abs promoted lysis of vibrio cells in vitro in the presence of guinea pig complement [58,64,70,71]. Studies with human volunteers showed that primary vibriocidal responses seemed to correlate better with IgM titres than with IgG ones [71]. It was however believed that IgA and IgG, rather than IgM, played a role in protection against cholera [49,51,54,65,69]. Colonization of the intestine by *V. cholerae* evoked a mucosal immune response in the host, including secretion of IgA antibodies (sIgA) that were thought to be involved in limiting the duration of the primary infection and in imparting resistance to subsequent oral challenge [42,61,62]. The response included polyclonal sIgA Abs directed against both CT and LPS [42,61]. Apter et al. [43] demonstrated that anti-LPS sIgA was much more effective than anti-CT sIgA in prevention of *V. cholerae*-induced diarrheal disease in suckling mice. Although IgA or IgG could be induced by *V. cholerae* LPS, the difficulty of generating these generally T cell-dependent Abs might contribute to cholera vaccine failure rates. Exposure to *V. cholerae* in the form of either infection or vaccination with intact bacteria could induce protective Ig characteristic of both T cell -independent (IgM) [72] and T cell-dependent responses (IgG and IgA) [49,51,72]. Experimental manipulation of the anti-LPS immune responses so that secretory IgA or IgG specific for *V. cholerae* O-PS are optimally induced will facilitate development of an effective cholera vaccine.

Gupta et al. [46] showed that conjugates prepared by binding of hydrazine-treated LPS from *V. cholerae* O1, serotype Inaba, to CT were safer and induced both serum IgM and IgG Abs with vibriocidal activity and IgG anti-CT. These authors suggested how serum vibriocidal Abs might prevent cholera. Serum Abs, especially those of the IgG class, penetrate into the lumen of the intestine; it is likely complement proteins are also present. The walls of the intestine then get in contact due to peristalsis. The inoculum of the vibrios that survive the gastric acid is probably low and the organisms have short polysaccharides on their LPS; this trait is associated with a high susceptibility to the complement-dependent action of serum Abs; the ingested *V. cholerae* are lysed on the intestinal mucosal surface [46].

The role of complements has, however, been debated for over years. Earlier, it was found that while *V. cholerae* O1 was readily and reliably lysed by complement in the presence of specific Ab, this was not the case with O139 strains because of the presence of capsular layers. Attridge et al. [73] devised a modified assay system and showed that the O139 strains were lysed by the Ab and complement. They observed that the earlier assay method and not the capsule production provided the major impediment to lysis by Ab and complement. Other workers [74–76] had questioned the role of complement in the protection effect of specific Ab against *V. cholerae* at least in the infant mouse cholera model (IMCM). They showed that the enzymic fragment $F(ab')_2$ of the IgG molecules retained the full protective activity despite losses in complement fixation. They proposed that the protection offered by the Ab was by cross-linking of bacteria and there by reducing the number of organisms adsorbed to the intestinal wall. Subsequently, Attridge et al. [77] showed that Mabs prepared against TCP

isolated from *V. cholerae* O1 El Tor were able to provide biotype-specific protection against experimental cholera in infant mice, although the Mabs were not lytic in the presence of complement. They suggested that Abs to TCP protected by directly blocking colonization of the mucosal surface rather than any complement -dependent lysis. But Kaper et al. [28] observed that long-term protection against cholera could be accomplished even in the absence of a detectable anti-TCP immune response. In the context of all these observations, the exact role of complements in the protection against cholera remains unclear.

4.3. Immunoglobulin subclasses

The Igs, IgA and IgG, have subclasses, which are known to exhibit different functions. Knowledge about the subclass distribution of specific antibodies in infection caused by *V. cholerae* O1 or O139 is limited. Jetborn et al. [78] had shown that CT induced responses of the four Ig G subclasses (IgG1, IgG2, IgG3, IgG4) and the IgA1 subclass in serum of the cholera vaccinees and patients. A study of North American volunteers had shown that secondary challenge with *V. cholerae* O1 resulted in LPS-specific responses of the IgG1 and IgG3 subclasses [71], where as after primary exposure, the major response to LPS was of IgG4 Abs. The LPS-specific IgG1 and IgG3 responses in the North American volunteers were highly associated with the vibriocidal activity in the IgG fraction, suggesting that these subclasses might also contribute to vibriocidal Abs. Qadri et al. [49] made a comparative study of the subclass distribution of the mucosal and systemic Ab responses in patients infected with *V. cholerae* O1 or O139 to two Ags, LPS and CT. They assessed the Ab secreting cells (ASC) in the circulation, which served as proxy indicator of the mucosal immune response. LPS-specific ASCs of both IgA1 and IgA2 subclasses were found, with the IgA1 ASC response predominating in both *V. cholerae* O1 and O139-infected patients. Both groups of cholera patients showed significant increases in LPS-specific IgG1, IgG2 and IgG3 Abs in plasma. Again, both groups of patients showed CT-specific ASC responses of the different IgG and IgA subclasses in the circulation. The authors showed that despite possessing a capsule and an LPS structurally different from that of *V. cholerae* O1, *V. cholerae* O139 induced Ab subclasses similar to those seen in O1 cholera. Further investigations are required to decide whether the LPS-specific response in the different subclasses can be used as an alternative marker of immunity and whether the vaccines against O1 and O139 cholera can be developed to stimulate Ab subclasses that are likely to offer protection.

4.4. Antibody assay for encapsulated cells

Recent studies evaluating the usefulness of the vibriocidal assay for O139 infections have produced conflicting results and strain-to-strain variability in the sensitivity of the vibriocidal assay to fully encapsulated O139 strain has been reported [48,79,80]. The possibility that the CPS might interfere with complement-mediated killing of the organisms prompted some workers to develop modified vibriocidal assay methods. Losonsky et al. [48] established a modified vibriocidal assay using another O139 target strain, strain 2 L, an unencapsulated insertion mutant of parent strain AI-1837, which retained the truncated O side chain. But the modified vibriocidal assay for fully encapsulated *V. cholerae* O139 strain AI-1837 and for the unencapsulated insertion mutant strain 2 L produced a very modest vibriocidal response in volunteers challenged with *V. cholerae* O139 that is not specific. Boutonnier et al. [81], on the other hand, prepared a conjugate of the polysaccharide moiety (O-specific polysaccharide plus core) of the LPS of *V. cholerae* O139 (pmLPS) and tetanus toxoid (TT) and tested its immunological properties using BALB/c mice. The conjugate (pmLPS-TT) elicited high levels of IgG antibodies, peaking 3 months after the first immunization and declining slowly during the following 5 months. Antibodies elicited by the conjugate recognized both CPS and LPS from *V. cholerae* O139, were vibriocidal and were protective in neonatal mouse model of cholera infection. The authors claimed that conjugation of the O139 pmLPS enhanced its immunogenicity and conferred T-dependent properties to this polysaccharide. Boutonnier et al. [82] further developed a new method (microtiter plate assay) for determining the vibriocidal Ab titer, which was considered equally convenient and efficient for both *V. cholerae* O1 and O139 serogroups. Their method was also found convenient for measuring the activity of animal sera and mouse MAbs.

A new assay using blocking of the limulus amebocyte lysate (LAL) reaction in a microtiter plate was developed [83] to detect Abs to *V. cholerae* O139 LPS that would be less susceptible to the confounding effects of the capsule. It was shown that Abs to *V. cholerae* blocked the LAL reaction and that LAL titers were comparable to the vibriocidal titers. Also blocking of the gel reaction was serotype specific. However, practical use of this method in field studies required further investigations. Nandy et al. [84], in a different approach, raised antisera to the truncated form of O-polysaccharides (TFOP) linked to the core of O139 LPS and found that anti-TFOP Abs and their Fab (IgG) fragments induced passive protection against challenge with colonial variants of encapsulated O139 strains in the suckling mouse model of experimental cholera. The authors found that such protection was mediated by inhibition of intestinal colonization.

4.5. Synthetic oligosaccharides

The use of a synthetic, O-PS-based immunogen was found to eliminate the toxicity problems associated with native LPS. Chernyak et al. [85] prepared immunogens by linking of BSA to the chemically synthesized, linker- equipped hexasaccharide fragment of the O-PS of *V. cholerae* O1, serotype Ogawa, by appropriate chemical methods [86,87] (Fig. 2). Conjugates with different carbohydrate (CHO)-to-carrier (BSA) molar ratios were tested for immunogenicity and efficacy in mice. All the conjugates tested were found to be immunogenic and a correlation was found between vibriocidal activity and protec-

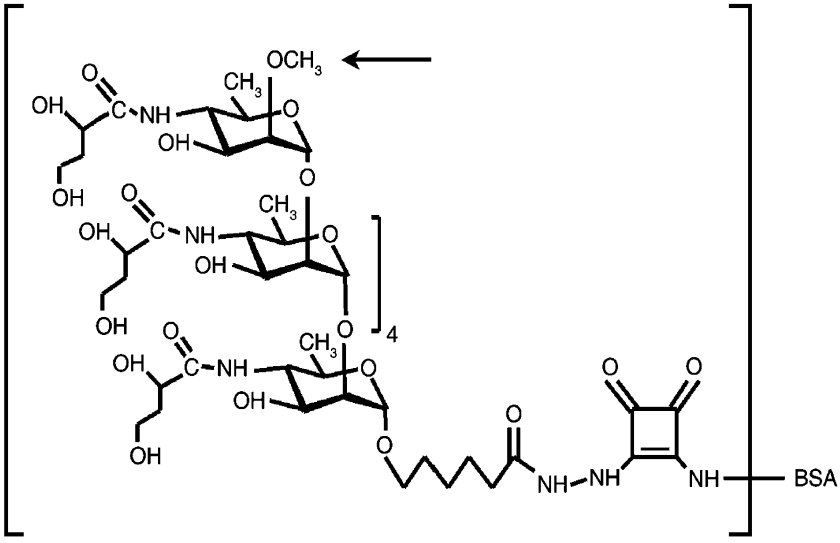

Fig. 2. Chemical structure of the neoglycoconjugate immunogen (CHO [Ogawa terminal hexasaccharide]–BSA) complex, as obtained from the works of Chernyak et al. [85,86]. BSA was linked to the chemically synthesized, linker-equipped hexasaccharide fragment of the O-PS of *V. cholerae* O1 serotype Ogawa. Arrow shows the 2-*O*-methyl group in the terminal sugar of the Ogawa serotype, which is replaced by 2-OH group in the Inaba serotype. Three different immunogens (A, B and C) based on the synthetic Ogawa epitope that varied in number of hexasaccharide residues and were covalently coupled to BSA were used to test immune responses in female BALB/c mice. The CHO to BSA molar ratios in the three immunogens were: A, 15.5:1; B, 9.2:1; C, 4.6:1.

tion. The protective capacity of antiserum was evident in serum from mice immunized with all conjugates, but it was highest in the groups that received the conjugate with the lowest level of substitution (conjugate C). The corresponding mice received fewer immunizations with conjugate C. The level of substitution and the number of immunizations affected the repertoire profile of the anti-Ogawa epitope response but the reasons for this differential protection were not known and required further investigations. Subsequently, a series of conjugates made from Inaba di-, tetra- and hexasaccharide and BSA were prepared [88,89] and found to be immunogenic in mice, inducing IgM and the T-dependent IgG1 subclasses. But the Inaba-specific Abs, IgM and IgG1, were neither vibriocidal nor protective in the infant mouse cholera model [89]. Again the exact reason for the functional differences between the anti-Inaba and anti-Ogawa Abs remained to be explained. In contrast to the anti-Inaba CHO–BSA sera, the secondary, anti-whole LPS sera were vibriocidal. The authors thus suggested that the Abs induced by the Inaba CHO–BSA conjugates did not bind with enough affinity or specificity to native LPS when expressed on the bacterial surface.

5. Role in the intestinal adhesion and virulence of the vibrios

Several studies [44,46,52,90] implied that the O-Ag represented a protective Ag and was involved in the adherence and colonization of *V. cholerae*. Fuerst and Perry [91] demonstrated LPS on sheathed flagella of *V. cholerae* O1 by protein A-gold immunoelectron microscopy. The flagellum on *V. cholerae* cells was found essential for in vitro attachment and enhanced initial colonization of the host intestinal surface in the infant mouse cholera model [92]. The LPS on the flagellum was thus found to function as a carrier of adhesins [93,94]. On the other hand, Mukhopadhyay et al. [95] observed that, in the mouse model, the anti-LPS Abs induced passive protection through microagglutination and/or immobilization of vibrios, which did not allow the vibrios to adhere to and colonize the intestine. The different animal models or cultured cells may, however, not be completely suitable to evaluate the factors that are essential for colonization of the human gut. To get a more realistic picture, Benitez et al. [96] studied the interactions between *V. cholerae* O1 and O139 with the highly differentiated mucin-secreting cells, HT29-18N2, which were derived from the human colonic adenocarcinoma HT29 cell line. Choleragenic vibrios were shown to adhere to and multiply on monolayers of these cells. Their adherence was partially inhibited by LPS. The authors further showed that the flagella, an active *toxR* gene, and the virulence cassette were not essential for binding. The authors emphasized that the interactions studied mimicked important events accompanying intestinal colonization and as such provided a new approach for studying factors involved in the intestinal colonization of the vibrios.

Several studies correlated LPS mutations with colonization defects in *V. cholerae* [97,98] and other organisms [99–101], but the mechanism by which LPS mutations decreased colonization remained unclear. Iredell et al. [102] studied some *wbe::Tn* mutants (which were resistant to phages known to use the O-Ag as their receptor) and tried to explain the role of LPS in virulence of *V. cholerae* O1. The authors found that the mutants were unable to assemble TCP on their surface, but the major subunit TcpA could be found as an intracellular pool. These mutants could be complemented back to wild type using the cloned *wbe* region implying that the functional TCP assembly was dependent upon an intact LPS. This was significant in the background of the finding that TCP was a protective Ag in animal models [103–105] and has been

shown to be an essential colonization factor for both human and infant mouse, and for both classical and El Tor strains [103,105,106]. While searching for genes required for colonization, Chiang and Mekalanos [107] identified *manB* and *wbeL* mutants as colonization-defective strains. They found no defect in the TCP production of *wbe* mutants or could detect any reduction in the TCP expression in a *gmd* :: *Tn 5lac* mutant of O395 strain. The authors accordingly concluded that the colonization defect associated with *wbe* mutations was unrelated to defects in TCP assembly but suggested, on the other hand, that LPS itself was important for colonization. They argued that although the exact role of LPS in colonization was unclear, the possibility that LPS defects might render bacteria more susceptible to gut-associated bactericidal substances appeared sensible particularly since LPS was known to be involved in resistance to antibiotics and complement mediated killing [108,109].

Nesper et al. [110] isolated bacteriophage K139-resistant mutant of *V. cholerae* O1 El Tor having intact O-Ag but altered core oligosaccharide and also a mutation in the *galU* gene. They further isolated another *gal* mutant (inactivated *galE*), which was defective in the catabolism of exogenous galactose but synthesized an apparently normal LPS. They found that the *galU* and a rough LPS mutant (R-LPS), but not the *galE* mutant, were defective in colonization, a phenotype also associated with O-Ag-negative mutants. Their study further showed that *galU* and R-LPS mutants were more sensitive to short chain organic acids, cationic antimicrobial peptides, the complement system, the bile salts and other hydrophobic agents, indicating that the outer membrane of these organisms could not provide an effective barrier function. The O-Ag-negative strains were also found to be sensitive to complement and cationic peptides, but displayed significant resistance to bile salts and short chain organic acids. This study indicated the involvement of *galU* in *V. cholerae* virulence, correlated with the observed change in LPS structure, and a role for both *galU* and *galE* in the environmental survival of *V. cholerae*. In a more recent study, the authors [111] further investigated the role of LPS O-side chain and CPS of *V. cholerae* O139 in intestinal colonization by using genetically engineered mutants. Their results showed that the loss of LPS O-side chain or CPS resulted in approximately 30-fold reduction in colonization of the infant mouse small intestine. Their study further indicated that in so far as *V. cholerae* O139 strain was concerned, the presence of both LPS O-side chain and CPS was important during the colonization process. On the other hand, Attridge et al. [112] obtained the bacteriophage JA-1 (which uses the capsule as the receptor)—mutants having several phenotypes, with loss of capsule and/or O-Ag from the cell surface, studied their residual complement resistance and infant mouse colonization potential and showed that production of O-Ag was of much greater significance than the presence of capsular material for both of the aforesaid properties. In the background of these studies, additional factors (other than the known colonization factors) involved in the colonization and acid tolerance of *V. cholerae* were subsequently identified [113]. Several genes were identified whose activity in colonization was not previously appreciated [113]. The functions of these genes included production of factors involved in metabolic activities, regulation of cellular processes, transport, adaptation to stress and some unknown functions. These authors identified nine new factors as crucial for the *V. cholerae* acid tolerance response (previously identified to be important for epidemic spread of cholera) and showed that mutations in the genes, *gshB*, *hepA* and *recO* resulted in a 1000-fold reduction in colonization [113].

6. LPS as phage receptor

V. cholerae LPS, like the LPS of many Gram-negative bacteria, was found to act as the receptor of several cholera phages [114–116]. Mukherjee's group IV cholera phage differentiates the classical and El Tor biotypes of *V. cholerae*, the classical ones being sensitive and the El Tor ones resistant to these phages [117]. The basis of this differentiation was traced down to the LPSs of the two biotypes. The phage φ149 was inactivated by the classical LPS but was resistant to the El Tor LPS [114,115]. Adsorption of cholera phage φ149 to isolated classical LPS followed a first order reaction kinetics, the 50% phage inactivating concentration of LPS (IC50) being 7 μg/ml. After treatment of LPS by 0.5% (w/v) sodium deoxycholate at 37 °C, the LPS largely lost its phage inactivating capacity and the IC50 value rose to 3.6 mg/ml [114,115]. This was in conformity with the fact that sodium deoxycholate dissociated LPS of Gram-negative bacteria into very small units with subsequent loss of biological activity [118,119]. When cholate was removed by extensive dialysis, the phage inactivating capacity of LPS was restored significantly, the IC50 value being 570 μg/ml of LPS. The cholate alone could not inactivate the phages by any significant degree [114]. LPS isolated from Inaba or Ogawa serotypes and classical or El Tor biotypes of *V. cholerae* showed identical phage inactivating capacities for the phage CP-T1 [116]. On the other hand, LPS from a CP-T1 -resistant mutant exhibited no phage inactivating capacity. The mutant was shown to lack the O-Ag by bactericidal assays utilizing a MAb directed against O-Ag side chain of *V. cholerae* LPS. The absence of O-Ag in the phage-resistant strain was further confirmed by SDS-PAGE study of ^{32}P-labelled LPS [116]. Similarly, another phage, VCII, specific to O1 classical strains found their receptors in the O-Ags of LPS and the VCII resistant mutants lacked the O-Ag [35].

Bacteriophage K139 was originally isolated from a *V. cholerae* O139 strain and was identified as belonging to the Kappa phage family [120]. Further analysis revealed that this phage was widely distributed among clinical El Tor strains and was also found as a defective prophage in classical O1 strains [120,121]. K-139 was perhaps the first vibriophage for which the entire genome was sequenced [122]. The tail fibers were thought to be involved in receptor binding. The presumed tail fiber genes of the phage K-139 were sequenced and analyzed, and two conserved and two variable regions were identified. Three different tail fiber types were discovered depending on the different combinations of the variable regions. Since the C-

terminal part of the tail fiber was believed to be involved in receptor binding [122], it was speculated that the variable regions of the K-139 phages determined their binding ability to different O-Ag receptors. Phage binding studies with purified LPS of different O1 serotypes and biotypes revealed that the O1 O-Ag served as the phage receptor. Analysis of the LPS of spontaneous phage-resistant mutants revealed that most of them synthesized incomplete LPS molecules composed of either defective O1 O-Ag or core oligosaccharide [121]. Applying hypervirulent phage K139cm9 to O1 El Tor strains, different phage-resistant mutants were isolated and these were found to express different LPS mutations. Interestingly several mutants were found linked not with the O1 O-Ag but with the core structure. Such mutants indirectly implicated the core region of the LPS in secondary phage infection steps [121]. Among the O-Ag defective mutants, one mutant was characterized for the loss of O-Ag due to transposition of IS1004 into the *wbeW* gene encoding a putative glycosyltransferase. In a later study [123], one *wbeW*: IS1004 serum-sensitive mutant was treated with normal human serum and several survivors showing precise excision of IS1004, restoring O-Ag biosynthesis and serum resistance were detected. Further, by screening for phage resistance among clinical isolates and performing LPS analysis of non-lysogenic strains, one strain was identified with decreased O-Ag presentation and significant reduction in ability to colonize the mouse small intestine. Several other cholera phages were identified as having receptors not in the cell wall LPS but in other structures associated with the organism [124–126]. Any discussion on these phages and their receptors is beyond the scope of this review.

7. Biofilm formation and the structure of LPS

Biofilm formation by bacteria is of great importance in respect of their survival in natural environments and causation of epidemic outburst of the disease. Biofilm can develop on abiotic surfaces and generally consists of bacterial cells entwined in a protective matrix of extracellular polysaccharides. *V. cholerae* is a natural inhabitant of aquatic ecosystems and is known to attach to different environmental surfaces. Adhikari and Chatterjee [127] reported the formation of thick pellicle on the surface of static liquid cultures of several mannose-sensitive haemagglutinating strains of *V. cholerae* El Tor and found a direct correlation between the formation of a special type of pili on the bacterial surface and pellicle formation. Tweedy et al. [128] confirmed the presence of pili on *V. cholerae* surface and produced evidence that the Vibrio strains, which exhibited weaker haemagglutination reaction, were comparatively poorer in pili formation. In the recent days, *V. cholerae* El Tor has been reported to form three-dimensional biofilm on abiotic surfaces [110,129–132] and on simple static liquid cultures [133] in agreement with the earlier observations of Adhikari and Chatterjee [127]. *V. cholerae* O1 El Tor N16961 required the MSHA, a type IV pilus, and the flagellum to associate with abiotic surfaces [129–131] in LB broth, where as *V. cholerae* O139 strain M010 depended only on the flagellum for surface association [132]. For subsequent development of a three dimensional biofilm, both the strains required the presence of the *vps* genes, which are responsible for the synthesis of an exopolysaccharide-based adhesive extracellular matrix [129,132,133]. Watnick and Kolter [132] further reported, using transposon mutagenesis, that the genes involved in biofilm formation included those encoding (i) the biosynthesis and secretion of the type IV pilus (MSHA), (ii) the synthesis of exopolysaccharide and (iii) flagellar motility. Accordingly, they suggested that the three steps in the process of biofilm formation were: (i) the type IV pilus and the flagellum accelerating attachment to the abiotic surface, (ii) the flagellum mediating spread along the abiotic surface and (iii) the exopolysaccharide forming the three-dimensional biofilm architecture. The exopolysaccharide initially forms the so-called slime layer on the surface of bacteria. The biofilm formation is normally associated with the change from normal smooth colony morphology to a rugose one of the bacteria [134]. The rugose colony morphology was the result of increased synthesis of the VPS exopolysaccharide [133,135], and transcriptional regulation of the *vps* genes, which are required for the synthesis of the VPS exopolysaccharide, was altered in these strains [136]. Thus, these variants rapidly formed biofilms in LB broth that were much thicker than those formed by smooth-colony variants of *V. cholerae*. Electron microscopic examination of the rugose form *V. cholerae* El Tor strain TSI-4 revealed thick electron dense exopolysaccharide materials surrounding polycationic ferritin-stained cells, while the ferritin-stained material was absent around the translucent form of the strain TSI-4. Scanning electron microscopic examination further revealed that the surface of the biofilm was colonized by the actively dividing rod shaped cells. By having exopolysaccharide materials, the rugose strains acquired resistance to osmotic and oxidative stress and were capable of causing human disease [137]. *V. cholerae* O139 strain M010 was also shown to produce exopolysaccharide leading to biofilm formation in response to nutrient starvation with concomitant change from a normal smooth colony morphology to a rugose one [134]. It was further demonstrated by immunoelectron microscopy that there was an epitope common to the exopolysaccharide Ag of *V. cholerae* O1 strain TSI-4 (rugose form) and that of O139 strain MO10 [134].

The entire *V. cholerae* O1 genome sequence being available [138], a method was developed for the whole genome characterization of the biofilm phenotype through the use of microarray-based expression profiling [139]. The important objectives of study were the differential expression pattern between the sessile and planktonic populations of the same culture, the identification of genes selectively expressed during different stages of biofilm development, identification of genes differentially expressed during adaptation of a mature biofilm to various changes in the fluid phase, etc. Hango and Watnick [140] subsequently identified a transcriptional repressor in *V. cholerae* that inhibited exopolysaccharide synthesis and biofilm development. It was shown that the repressor was the *V. cholerae* homologue of *E. coli* CytR, a protein that represses nucleoside uptake and catabolism when nucleosides are scarce.

The influence of biofilm formation on the structure of LPS or vice versa among *V. cholerae* cells is likely to form another important field of study. In *Pseudomonas aeruginosa*, studies had indicated that changes in LPS phenotype affected adherence properties and influenced biofilm formation [141]. Recently, Nesper et al. [110] studied several aspects including the resistance to phage K139.cm9 of and biofilm formation by the different *gal U* and *gal E* mutants of *V. cholerae* El Tor. Among the spontaneous phage K139.cm9-resistant strains, they found strains with a rugose colony morphology constitutively synthesizing an exopolysaccharide and producing biofilm on abiotic surfaces. They introduced *galU* and *galE* mutations into the rugose variant P27459res105 and found that both mutations yielded smooth colony forms, suggesting that *galU* and *galE* mutants were unable to synthesize the exopolysaccharide and could not form the biofilm. The activated carbohydrate moieties, like UDP-glucose and UDP-galactose, were often involved in the synthesis of different surface structures of bacteria [110]. Enzymes for the biosynthesis of UDP-glucose and UDP-galactose are UDP-glucose-pyrophosphorylase, encoded by *gal-U*, and UDP-glucose-4-epimerase, encoded by *galE* [142]. The fact that *galU* and *galE* were found essential for the formation of a biofilm by the phage-resistant rugose variant suggested that the synthesis of UDP-galactose via UDP-glucose was necessary for the biosynthesis of exopolysaccharide. Kierek and Watnick [143] reported recently the formation of *vps* -independent biofilm of *V. cholerae* in model seawater [144]. Although Ca^{2+} was shown to be required for the formation of *vps*-independent biofilm, the exact mechanism underlying the Ca^{2+} dependence of *vps*-independent *V. cholerae* biofilm formation has not yet been established. It was, however, shown that (i) both MSHA and flagellum were required for the formation of vps-independent biofilm of *V. cholerae*, (ii) both *V. cholerae* O139 O-Ag and capsule promoted this biofilm formation, (iii) spontaneous unencapsulated variants of *V. cholerae* O139 also exhibited markedly increased surface association, (iv) Ca^{2+} was an integral component of the *vps*-independent extracellular biofilm matrix and (v) the *V. cholerae* biofilm formed in true sea water exhibited O-Ag polysaccharide-dependence and disintegrated upon exposure to true fresh water (Fig. 3). LPS was thus found to play a significant role in *V. cholerae* biofilm formation.

8. Capsular polysaccharide (CPS)

Both LPS and CPS of the strain O139 were found immunogenic. They reacted in an enzyme immunoassay with

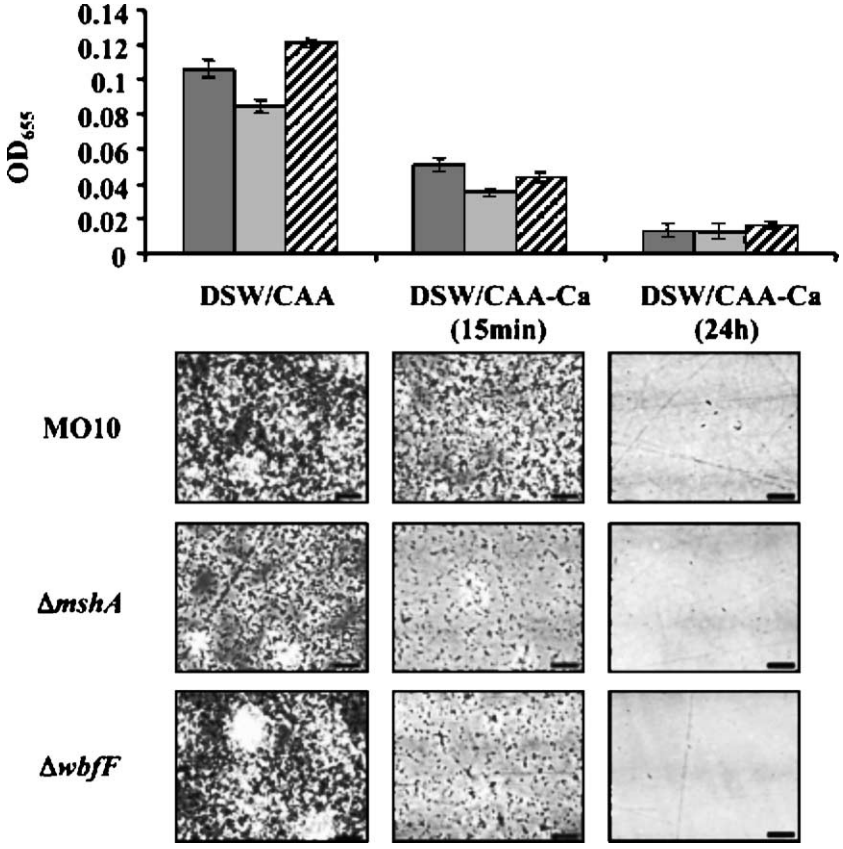

Fig. 3. Quantification (upper) and phase contrast microscopy (lower) of wild type *V. cholerae* (M010; black bars), ΔmshA mutant (grey bars) and ΔwbfF mutant (striped bars) biofilms after incubation in DSW medium (including casaamino acids, CAA) for 24 h and then 15 min and 24 h after replacement of bathing medium with DSW medium lacking Ca^{2+} (−Ca) [143,144]. DSW medium is a defined medium of salts based on the composition of artificial sea water [144]. ΔwbfF mutant has transposon insertion in gene responsible for export of capsule precursor and ΔmshA mutant is the one harboring deletion in the gene *mshA* The figure illustrates how the biofilms formed by the wild type and the mutants of *V. cholerae* disintegrated rapidly after removal of Ca^{2+} from the medium. Reproduced from the paper of Kierek and Watnick [144] with permission from PNAS, USA.

rabbit Abs generated against heat-killed bacteria [145]. Waldor et al. [97] carried out immunoblot analysis of either whole cell lysates or LPS preparations and obtained three electrophoretic forms of the O139 Ag, i.e., two slowly migrating forms and one rapidly migrating one that appeared identical to O139 LPS. All three forms of the Ag shared an epitope defined by an O139 specific MAb. A serum-sensitive non-encapsulated mutant was isolated that lacked only the slow migrating forms. The slow migrating forms did not stain with silver whereas the rapidly migrating forms did, indicating that the former might constitute highly polymerized O-Ag side chain molecules that were not covalently bound to core OS and lipid A, i.e., the O-Ag capsule. This is in conformity with the observations of other workers [145,146] that the *V. cholerae* O139 serogroup Ag includes both the LPS and the CPS.

The presence of capsule on *V. cholerae* O139 strain contributed to its virulence in several ways. The CPS made the strain more resistant to killing by normal human serum and the loss of capsule was associated with loss of the resistance [147]. Johnson et al. [148] derived an unencapsulated mutant of O139 strain by transposon mutagenesis and showed that it was readily killed by serum, while the encapsulated one was protected significantly. The unencapsulated mutant was less virulent in a mouse model than the encapsulated parent. Another factor contributing to the virulence of the non-O1 strains of *V. cholerae* in general was septicemia [148]. Non-O1 *V. cholerae* strain NRT36S produced a polysaccharide capsule that determined colony morphology, serum resistance and virulence in mice. The causation of such extraintestinal disease has not been found with *V. cholerae* O1 strains. A third reason for the increased virulence of the encapsulated strains of *V. cholerae* vis-à-vis the nonencapsulated ones was that both the LPS and CPS were important for their colonization of the small intestine of the new born mouse [146]. There was also evidence that the CPS mediated adherence to epithelial cells [97]. Using the intestinal epithelial cell line Caco-2, a clear correlation between the amount of capsular material expressed and the avidity of binding to Caco-2 cells was found. In view of all these findings, Johnson et al. [145] observed that the presence of capsule on O139 strains had profound implications for vaccine development. There was already a report of septicemia caused by an O139 strain [149]. Further, in keeping with observations with other non-O1 isolates [150], sepsis was found to occur in a patient with underlying liver disease. Since O139 strains might follow the pattern of other non-O1 strains, the risk of dissemination would be greatest in persons with chronic underlying illness [150,151]. Thus, the advisability of administering oral attenuated vaccines that still carry the capsule to persons who may have underlying illness may be questioned. Further works addressing these problems will be of practical importance.

9. Concluding remarks

9.1. Recognition of LPS and activation of host innate immunity

The molecular mechanisms involved in the recognition of LPS of Gram-negative organisms and the initiation of host response have been reviewed recently [152–154]. At the extracellular stage, LPS has to be bound to a transport molecule, a lipid-binding protein (LBP), which facilitates its binding to a surface protein, CD14. CD14 then brings LPS to the proximity of the cell membrane. The LPS-binding protein, MD-2, then opsonizes LPS to be recognized by another protein, TLR4, for initiation of signal transduction. LPS is then briefly released into the lipid bilayer where it interacts with a complex of receptors, e.g., heat shock proteins (HSPs) and others, depending upon the cell type. TLR-mediated signaling activates signal transduction pathways (such as NFkβ, JNK/p38, NF/IL6 and IRF) that induce transcription of cytokines (such as TNF-α and the type 1 interferons) and that in turn stimulate immune function and control expression of a variety of inducible immune response genes. A recent study has shown that *V. cholerae* LPS acts through the TLR 4-MyD88-dependent signaling pathway and induces INF-α, IL-1β and MIP-3α and significantly lesser amounts of IFN-β, nitric oxide and IP-10 in macrophages [155]. Further studies on *V. cholerae* LPS are required at least for having a better knowledge of its interaction with the B cells involving TLRs.

9.2. Serogroup surveillance and monitoring

The structure of the O-PS of *V. cholerae* of any serogroup has been found to be unique [1]. The genetic organization encoding the O-PS biosynthesis is quite susceptible to change, but the factors responsible for effecting such changes are still largely unknown [2]. Thus, a new serogroup or any of the known serogroups may acquire pathogenic potential in epidemic genetic background and may cause future epidemics. This situation demands a continuous and strict surveillance and monitoring of the emergence of either a new serogroup or any of the known serogroups with pathogenic potential so that appropriate vaccines can be devised promptly.

Phages are known to play a role in the emergence of pathogenic clones and may also be involved in territorialism between different strains of *V. cholerae*. For example, CT genes were transferred to non-toxigenic strains through a lysogenic filamentous phage [126], and the emergence and dominance of *V. cholerae* O139 in Bangladesh and India during 1992–1993 might have involved phages both as a means of horizontal gene transfer as well as a bacteriocidal selective mechanism. Faruque et al. [156] monitored the environmental water samples of Bangladesh for nearly a 3-year period and found that significantly more of these samples contained either a phage targeting *V. cholerae* LPS as its receptor or a phage-susceptible *V. cholerae* strain than both. Interepidemic periods were characterized by water samples containing cholera phages but no viable bacteria. Faruque et al. [157] further observed that host-mediated phage amplification during the cholera epidemic likely contributed to increased environmental phage abundance, decreased load of environmental *V. cholerae*, and, hence, the collapse of the epidemic. The authors thus put forward the important suggestion that environmental surveillance for vibriophages could be useful in

tracking outbreaks, predicting epidemics and anticipating emergence of new serogroups. Further, vibriophages might also be employed as biological control agents in cholera epidemic areas [157].

But any effective surveillance and monitoring of the aqueous environments demands the availability of rapid diagnostic tests for cholera. Several such tests were available for *V. cholerae* O1 or O139 using LPS Ag [158–161]. A multistep colloidal gold-based colorimetric immunoassay known as SMART was also developed for direct detection of *V. cholerae* O1 or O139 in stool samples [162,163]. For rapid detection of *V. cholerae* O1 or O139, Nato et al. [164] described the development of a diagnostic test, the one-step immunochromatographic dipstick test, based on LPS detection using colloidal gold particles and immunochromatography. This test was claimed to be of very high specificity and sensitivity and could provide a simple tool for epidemiological surveys. Similar simple and rapid test should also be developed for the different serogroups of *V. cholerae*. Robert-Pillot et al. [165] devised a method for improved and specific detection of *V. cholerae* in environmental samples by culture of selective medium and colony hybridization assay with an oligonucleotide probe. The rapid detection of pathogenic *vibrios* using biochemical and immunological markers, PCR and DNA microarrays techniques will be a very challenging task. This will require use of DNA sequences specific for virulence genes or *vibrio* species to build and optimize a DNA microarray chip that specifically identifies pathogenic isolates of various *vibrio* species [166]. The successful development of such a technique will provide a less time-consuming diagnostic strategy to be used in the surveillance and monitoring of the estuarine or environmental water samples.

9.3. LPS and cholera vaccine

Since the day of isolation of *V. cholerae* by Koch in 1883, several cholera vaccines have been developed and evaluated in clinical trials [65,167,168]. The involvement of LPS O-Ag in the design and preparation of cholera vaccine using recombinant DNA technology and synthetic carbohydrate chemistry has been a rather recent and alternative approach. An attempt to construct cholera vaccine using recombinant DNA technology used the attenuated *Salmonella typhi* vaccine strain Ty21a containing cloned *V. cholerae* genes expressing the O-Ag [169–171]. Yet another novel idea for the design of a carbohydrate based cholera vaccine originated from the work of Villeneuve et al. [41] and which has been discussed earlier. Abs specific for the terminal perosamine could selectively protect against the Ogawa serotype but failed to recognize the Inaba serotype. Therefore, protective Abs against both serotypes should, the authors argued, bind to the inner part of the O-PS and/or sugar residues defining the core of the LPS molecule. The idea seems to be promising but remains to be implemented in practice. It was however proposed by another group of workers [172] that serum IgG Abs conferred protection against enteric diseases by inactivating the inoculum on the mucosal surfaces. At the level of laboratory animals, systemic administration of IgG Abs specific for the O-PS of *V. cholerae* O1 was found to protect neonatal mice against loss of weight and death following intragastral challenge with *V. cholerae* O1 [66]. It may be pertinent to note here the fundamental limitation that natural infection with *V. cholerae* does not occur in animals, although a few of the animal models have yielded useful information relevant to human disease. Further, the infant mice or the infant rabbits may be susceptible to infection with *V. cholerae* but only for a relatively short time after birth. It is therefore generally recognized that only the volunteer challenge studies with *V. cholerae* can give most useful information about the human disease.

Cellular cholera vaccines are poor immunogens and have T cell-independent properties [53,57,173]. Besides, LPS as a vaccine or in cellular vaccine often exhibits adverse reactions due to its endotoxic properties. The recipients of the cellular vaccine usually have high level of IgM anti-LPS Ab for about 6 months. The rapid decline of this IgM vibriocidal activity explains the short-lived protection conferred by cellular vaccines [52,70,174]. With a view to eliminating these undesirable properties of the cellular vaccines, two groups of workers have produced conjugate vaccines by coupling ‘detoxified’ LPS to protein carriers [46,83]. Gupta et al. [46] produced the deacylated LPS (DeALPS) by treatment of LPS with hydrazine there by reducing the endotoxic properties of LPS to clinically accepted levels. Conjugate vaccines were prepared by binding DeALPS from *V. cholerae* O1, serotype Inaba, to CT variants, CT-1 and CT-2 (used mainly as carrier proteins which are also immunogenic), with a spacer and evaluated (Phase I trial) in healthy volunteers [175]. The conjugates elicited the highest levels of IgG anti-LPS vibriocidal Abs, which persisted longer than those elicited by the whole cell vaccine. The authors, however, expected to improve further the level of IgG anti-LPS achieved with their conjugates so far. Although the pmLPS conjugate vaccine prepared by Boutonnier et al. [81], already discussed in Section 4.4 of this review, was found protective in the neonatal mouse model of cholera infection, it also remains to be evaluated clinically. An earlier study [176] showed that a *V. cholerae* O139 CPS-TT conjugate vaccine induced protection in rabbit ileal loop model of experimental cholera. Recently, *V. cholerae* O139 CPS conjugated with a recombinant mutant diphtheria toxin was shown to elicit high levels of serum anti-CPS IgG in mice with vibriocidal activity [72]. The use of chemically synthesized, linker-equipped hexasaccharide fragment of the O-PS of *V. cholerae* conjugated to BSA as an immunogen, as discussed earlier, has been very promising. But the use of these synthetic conjugates as vaccine candidates requires further investigation and clinical evaluation.

The goal of having a long-lasting vaccine effective against more than one serogroup and/or serotype of *V. cholerae* still remains to be achieved. Although the task ahead may be challenging, it certainly demands greater attention of researchers in the context of the long-standing global defence problem against the recurring cholera epidemics.

Acknowledgements

Authors are most thankful to the many scientists and particularly to Drs. Paul I. Watnick, P.M. Alzari, J.M. Fournier, P. Kovac, P.A. Manning, U.H. Stroeher and B.S. Srivastava for kindly sending reprints of their relevant publications and materials for our use and also to Dr. Diane Sullenberger, Executive Editor, PNAS, U.S.A. for granting permission to reproduce in this review article (i) Fig. 5 of the paper of Villeneuve et al. [41], copyright (2000) National Academy of Sciences, USA and (ii) Fig. 2 of the paper of Kierek and Watnick [144], copyright (2003) National Academy of Sciences, U.S.A. Sincere thanks are due to Dr. M. Maiti, Director-Grade Scientist, Indian Institute of Chemical Biology, Calcutta for helping us in many ways all through. Thanks are also due to Drs. Sanjay Nag and Raghunath Chatterjee of Biophysics Division, Indian Institute of Chemical Biology, for rendering technical help during the preparation of the manuscript.

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